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CD8⁺ T Cell Epitope-Flanking Mutations Disrupt Proteasomal Processing of HIV-1 Nef¹

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CTL play a critical role in the control of HIV and SIV. However, intrinsic genetic instability enables these immunodeficiency viruses to evade detection by CTL through mutation of targeted antigenic sites. These mutations can impair binding of viral epitopes to the presenting MHC class I molecule or disrupt TCR-mediated recognition. In certain regions of the virus, functional constraints are likely to limit the capacity for variation within epitopes. Mutations elsewhere in the protein, however, might still enable immune escape through effects on Ag processing. In this study, we describe the coincident emergence of three mutations in a highly conserved region of Nef during primary HIV-1 infection. These mutations (R69K, A81G, and H87R) flank the HLA B*35-restricted VY8 epitope and persisted to fixation as the early CTL response to this Ag waned. The variant form of Nef showed a reduced capacity to activate VY8-specific CTL, although protein stability and expression levels were unchanged. This effect was associated with altered processing by the proteasome that caused partial destruction of the VY8 epitope. Our data demonstrate that a variant HIV genotype can significantly impair proteasomal epitope processing and substantiate the concept of immune evasion through diminished Ag generation. These observations also indicate that the scale of viral escape may be significantly underestimated if only intraepitope variation is evaluated. *The Journal of Immunology*, 2005, 175: 4618–4626.

Cytotoxic T lymphocytes recognize endogenously generated peptide Ags bound to MHC class I molecules and play an essential role in host immune defense against intracellular pathogens. In HIV infection, rapid replication rates coupled with error-prone reverse transcription result in the generation of a large number of viral variants (1, 2). Within each host, infection is characterized by a large degree of viral genetic diversity, which facilitates rapid adaptive evolution in vivo. One well-recognized form of HIV adaptation is evasion of HIV-specific CTL activity (3–9). The genetic variation often involves mutations within viral antigenic epitopes, providing one of the basic mechanisms for escaping the host HIV-specific immune response. Epitope variants can interfere with CTL activity either through loss of binding to the presenting HLA class I molecule or through lack of recognition of the altered peptide by CTL TCR (10–14).

There are regions of the HIV genome that are highly conserved (15–20). Mutations within CTL epitopes in these regions could lead to the production of nonviable virions, and so impede viral

propagation. Tolerated mutations that lie outside the conserved sequence might enable immune escape through alternative means, such as disruption of epitope processing (reviewed in Ref. 21). Effects of the so-called flanking mutations on Ag presentation have been investigated through genetic manipulation of amino acid residues in the vicinity of the epitope. Changes in the epitope-flanking region can result in the inhibition of epitope presentation or a significant (up to 150-fold) increase in the generation of the epitope (22, 23). A C-terminal-flanking mutation in an influenza hemagglutinin epitope was shown to inhibit its presentation by impairing TAP-mediated transport of the peptide precursor (24, 25). Niedermann et al. (26) demonstrated that flanking regions also influence epitope hierarchy: reciprocal exchange of the flanking regions of a subdominant and dominant epitope from chicken OVA led to an inversion in the presentation levels of the two epitopes. The proposed mechanism for this phenomenon was the difference in the proteasomal cleavage patterns of the two constructs, highlighting the critical role of the epitope-flanking regions for proteasome function (26). Several studies have emphasized the importance of using full-length constructs when investigating Ag processing, because the same flanking sequences behaved differently when expressed within truncated minigenes (27–29). Although no general rules relating to the effect of flanking residues on epitope processing have been established, these findings collectively suggest that the generation of many HLA class I-restricted peptides is profoundly influenced by amino acid variation in and around epitopes.

The first example of a naturally occurring epitope-disrupting flanking mutation emerged in the field of tumor immunity, where a C-terminal-flanking mutation in the p53 protein abolished the presentation of the flanked epitope, thus protecting the tumor from CTL surveillance (30). Speculation arose that mutations at sites flanking viral epitopes could serve as a mechanism for immune evasion by altering Ag processing or TAP-mediated transport. A study using engineered strains of HIV containing randomly chosen

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mutations that flanked the HLA A*02-restricted Gag SL9 epitope found that most of them did not abolish the epitope-specific CTL response (31). Subsequently, epitope-flanking polymorphism was shown to impede correct epitope excision in murine Moloney virus (32). Two recent studies describe immune escape in HIV due to naturally occurring variation; one mutation caused erroneous trimming of the HLA B*57-restricted Gag epitope IW9 (33), the second mutation abolished CTL responses to two immunodominant HLA A*03-restricted Gag epitopes (34). Another study attributed the lack of recognition of endogenously presented epitope variants, which were recognized when presented exogenously, to peptide processing in infected cells (35). These findings suggest that the effects of mutations on epitope processing cannot be predicted and that immunological consequences of naturally occurring flanking variants must be investigated experimentally.

HIV-1 Nef protein is expressed at high levels early in HIV infection (36) and elicits a strong CTL response in many patients (37). Most antigenic determinants are located within a multirestricted, immunodominant central region spanning residues 73–94 and 113–147 (38–40). HIV-infected HLA B*35⁺ patients typically make a strong response to the VPLRPMTY epitope (residues 74–81), which lies within a highly conserved region critical for Nef function (41, 42). In this study, we characterized three mutations flanking the HLA B*35-restricted Nef VPLRPMTY (VY8) epitope. These mutations emerged during primary infection and were associated with the waning of the HLA B*35-restricted VY8-specific CTL response.

Materials and Methods

Patient

Patient SC1³ (seroconverter 1), a Caucasian homosexual male (HLA A*24, B*35/*55, Cw*1/*4) was admitted to the hospital with oral candidiasis and prolonged flu-like symptoms. At that time, HIV-specific Ab responses were detected. In a previous test, 7 mo earlier, SC1 was seronegative. Proviral *nef* DNA obtained from SC1 at two time points in the early course of infection was cloned and sequenced using a previously described method (12). Patient's PBMCs were isolated using standard Ficoll-Hypaque density gradient centrifugation (Axis Shield Diagnostics).

Construction of the recombinant vaccinia viruses (rVVs) *rVV-Nef-wt* and *rVV-Nef-mut*

rVVs used in this study were made by homologous recombination into the thymidine kinase gene of the plasmid pSC11 derivative (43), using a previously described protocol (44). A thymidine kinase-deficient cell line strain 143 (TK-143; European Collection of Cell Cultures) was used as a host in constructing two rVV constructs, one containing the proviral DNA *nef* sequence from a time point before the mutations (rVV-Nef-wt) and the other containing proviral sequence following the fixation of the three B35-restricted VY8 epitope-flanking mutations R⁶⁹K, A⁸¹G, and H⁸⁷R (rVV-Nef-mut). The rVVs were further amplified and titrated according to standard protocols (45). Construct fidelity was confirmed by PCR and full-length insert sequencing.

⁵¹Cr release cytolytic assay

A standard cytolytic assay was used as described previously (46). In this assay, target cells were infected with rVV for 90 min and allowed to express for an additional 3 h. These cells were then incubated with 100 μ Ci of ⁵¹Cr for 90 min at 37°C. For peptide-pulsed targets, synthetic peptide was either added afterward for 60 min and washed away or added directly to the CTL assay; both methods provided similar responses. Labeled cells were extensively washed and then placed at 37°C for an additional 30 min to allow for the removal of residual noninternalized chromium. Heterologous CTL clones specific for the HLA B*35 VY8 Nef epitope were used as effector cells. Serial 3-fold dilutions of the CTL were aliquoted in 100 μ l onto 96-well plates first. Labeled target cells (5000 or 10,000 cells) in

100 μ l were added to each well containing CTL. Spontaneous release of chromium was determined by analyzing the free counts (release) from target cells in only R10 medium. Total release of incorporated chromium was obtained from target cells treated with 5% Triton X-100 detergent. The 96-well plates were incubated for 4–16 h, analyzing multiple time points. Free counts were determined by carefully pipetting off 20 μ l of supernatant, transferring it to a Spot-On Filtermat (Wallac) and analyzing it with a 1205 Betaplate liquid scintillation counter (Wallac). Specific lysis was calculated as follows: $100 \times (\text{experimental lysis} - \text{spontaneous lysis}) / (\text{maximum lysis} - \text{spontaneous lysis})$.

Each experimental point was measured in duplicate and compared against quadruplicate controls. Overnight (16 h) chromium release CTL assays were only considered significant if the spontaneous release of ⁵¹Cr, which is typically 20% after a 4–6 h assay, was below 35%.

For CTL assays involving lactacystin, cells were treated with the drug for 45 min before infection. Pretreatment for 45 min with the irreversible proteasome inhibitor lactacystin was sufficient to block proteasome-mediated processing for at least 18–24 h. During some overnight assays lactacystin was maintained at 1 μ M (100 times less than the normal treatment).

Pulse-chase assay for protein stability

Labeling and immunoprecipitation of infected cells were performed as described previously (47). Briefly, methionine-starved fibroblasts were infected with rVV-Nef-wt or rVV-Nef-mut, allowed to express for 2–3 h, and labeled with [³⁵S]methionine (Amersham Biosciences) for 60 min. A standard pulse-chase assay was conducted, with chase time points of 0, 2.5, 5, and 20 h. Nef was immunoprecipitated using Nef-specific sheep antiserum ARP444 (National Institute for Biological Standards and Control, Medical Research Council Centralized Facility for AIDS Reagents, Hertfordshire, U.K.).

Anti-Nef immunoblotting

Cells infected with rVV-Nef-wt or rVV-Nef-mut were lysed on ice for 30 min in lysis buffer (140 mM NaCl, 20 mM Tris (pH 8.0), 10 mM sodium fluoride, 2 mM EDTA, 20% glycerol, 1% IGEPAL, 1 mM Na₃VO₄, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin), and the noncytoplasmic fraction was pelleted by centrifugation at $16,000 \times g$ for 15 min. The remaining lysate was aspirated and a small volume added to an equal volume of SDS loading buffer (350 mM Tris (pH 6.8), 350 mM SDS, 30% glycerol, 600 mM DTT, 175 μ M bromophenol blue). The sample was denatured for 4 min and run on a 15% SDS-PAGE protein gel. Before immunoblotting, the gel, filter papers (Bio-Rad), and nitrocellulose membrane (Amersham Biosciences) of matching size were equilibrated in ice-cold transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) for 10 min. Proteins were transferred from the gel onto the membrane by electrophoresis at 350 mA for 1 h. The gel was subsequently stained with Coomassie blue to verify transfer and equal protein loading. The membrane was blocked overnight in PBST (PBS/0.1% Tween 20)/0.5% milk, washed three times for 10 min in PBST, and incubated for 3 h with sheep polyclonal anti-Nef ARP444 Ab (dilution 1/1000) in PBST/0.5% milk. After three more washes in PBST, the membrane was incubated with mouse anti-sheep peroxidase-conjugated secondary Ab (Sigma-Aldrich), 1/2500 dilution in PBST/0.5% milk for 1.5 h. After three additional washes, the blot was developed using chemiluminescent substrate SuperSignal Pico (Perbio). All washes and incubations for Nef immunoblots were performed at 4°C. At least 48 h after development, blots were reprobated for actin to control for protein loading. To reprobe, the membrane was washed three times in PBST then probed with cross-species anti-actin rabbit Ab (Sigma-Aldrich; 1/500 in PBST/2.5% milk) for 1.5 h, washed, and incubated with secondary Ab (anti-rabbit-Ab, peroxidase-conjugated; Sigma-Aldrich), 1/10,000 dilution in PBST/2.5% milk for 1 h. The blot was washed three times in PBST and developed as described above. The incubations and washes for actin immunoblots were conducted at room temperature.

ELISPOT assay for single-cell IFN- γ release

Ag-specific responses were measured using a standard ELISPOT assay for IFN- γ , as described previously (48). CTL from clones and lines were tested in a 4-h assay, using an EBV-transformed B cell line (BCL) bearing HLA B*35 and HLA B*08 as Ag-presenting targets (30,000 BCL/well) and 500–1500 CTL/well. All assays were performed in duplicate or triplicate, and positive (PHA) and appropriate negative controls were included in every assay. Spots were counted using an ELISPOT reader system ELR02 (Autoimmun Diagnostika).

³ Abbreviations used in this paper: SC1, seroconverter 1; rVV, recombinant vaccinia virus; BCL, B cell line; TPP II, tripeptidyl peptidase II; DFOS, days following the onset of symptoms.

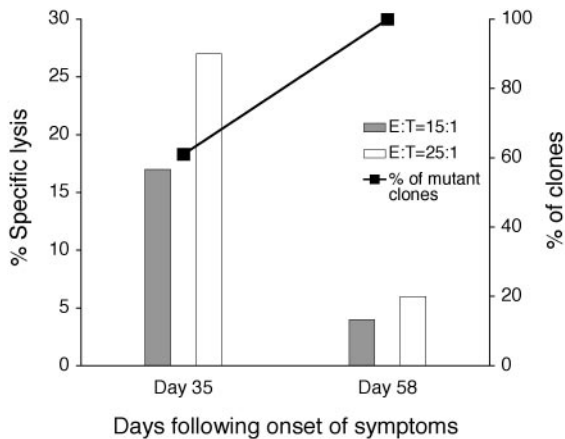


FIGURE 1. Temporal association of the loss of the HLA B*35 Nef VY8-specific CTL response in donor SC1 with the emergence of virions containing the three VY8 epitope-flanking mutations (R⁶⁹K, A⁸¹G, and H⁸⁷R). Ex vivo cytolytic response in SC1 was measured using the ⁵¹Cr-release method with synthetic VPLRPMTY peptide and autologous B cells as targets at different E:T ratios, as shown. Analysis of HIV-1 proviral sequences at 35 and 58 DFOS revealed progressive domination of virions carrying three epitope-flanking mutations: R⁶⁹K, A⁸¹G, and H⁸⁷R. From the total of 42 clones analyzed at 35 DFOS, 36% were found to have the wild-type Nef consensus sequence, and 64% possessed the three mutations. At 58 DFOS, all of the analyzed clones ($n = 19$) had the three flanking mutations ($p = 0.0013$, Fisher's exact test).

CTL lines and clones

The CTL lines and clones recognizing the HLA B*35 VY8 and HLA B*08 FL8 epitopes were generated from PBMCs of HIV-infected patients with detectable CTL responses to these epitopes. Lines were set up with corresponding synthetic peptides as described previously, with minor modifications (49). Briefly, ~3 million PBMCs were resuspended in 2 ml of R-10 medium (RPMI 1640, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10% FCS) containing 10 µM peptide and 25 ng/ml IL-7 (PeproTech) and cultured in a 24-well plate. On day 3, the medium was replaced with R-10 containing 200 U/ml IL-2 (Proleukin) and 5% T-STIM with PHA (BD Biosciences). The lines were subsequently grown in the same medium (and restimulated using irradiated mixed heterologous PBMCs and PHA) or maintained in R-10 containing 25 ng/ml IL-15 (PeproTech).

Intracellular proteasome inhibition assay

Inhibition of intracellular processing of the HLA B*35 VY8 epitope was tested using the proteasome inhibitors Epoxomicin (BIOMOL) and MG 132 (Merck), tripeptidyl peptidase II (TPP II) inhibitor AAF-CMK (BIOMOL), and cysteine protease inhibitor EST (Merck) with wild-type and mutant vaccinia in an ELISPOT assay. Approximately 10⁶ HLA B*35⁺ EBV-transformed B cells were treated with varying concentrations of each inhibitor in a 96-well U-bottom plate at 37°C for 45 min before addition of rVV at a multiplicity of infection of 5–10. The cells were then incubated for 90 min at 37°C in order for infection to occur. To control for any effects of the vaccinia infection itself, an rVV containing the influenza matrix gene

was used (50). Infected BCL were rested in 1 ml of R-10 medium overnight for Nef protein expression to occur before being used as targets in a 4-h ELISPOT assay; the target cells were added at 35,000 cells/well. The exception were cells treated with MG 132, a reversible inhibitor, which were rested in R-10 medium supplemented with MG 132 at the appropriate final concentration during Nef expression. For each inhibitor, four wells were set up without exogenous VY8 peptide (assay wells) and four wells with VY8 peptide at 1 µM final concentration (positive control wells).

Proteasomal digestion

In vitro proteasomal digestion of synthetic 25-mer oligopeptides (Biosynthesis) spanning the HLA B*35-restricted Nef VY8 epitope, corresponding to Nef-wt and Nef-mut sequences, was conducted with reference to a previously described method (51). For each oligopeptide, 5 µg of peptide and 2 µg of the constitutive-20S (c20S) or immuno-20S (i20S) proteasome (purified from human cells or cell lines; Immatics) were added to 300 µl of buffer (20 mM HEPES/KOH (pH 7.8), 2 mM MgAc₂, 2 mM DTT) and incubated at 37°C. Aliquots of the reaction mix were taken at several time points (0, 4, 6, 12, 18, and 24 h), and the reaction was terminated with acetic acid (10% final concentration). Digests were then analyzed by Mass Spectrometry (Ettan MALDI ToF; Amersham Biosciences), and sequences were inferred using Protein Analysis Work Sheet (<http://bioinformatics.genomicsolutions.com/paws.html>). To check for any nonspecific peptide degradation, control reactions without the proteasome were performed for each peptide. A proteasome efficacy control assay containing 10 µM of the proteasome inhibitor Epoxomicin (BIOMOL) was also conducted.

Results

Emergence of the flanking mutations coincided with the loss of the CTL response to VY8 in patient SC1

Using a synthetic peptide, we detected cytolytic activity against the HLA B*35-restricted Nef epitope VPLRPMTY (VY8) ex vivo in patient SC1 35 days following the onset of symptoms (DFOS) of seroconversion. Three weeks later (58 DFOS), and subsequently, no HLA B*35-restricted Nef-specific CTL activity could be detected (Fig. 1). Sequencing of HIV-1 *nef* proviral DNA showed no variation within the HLA B*35-restricted VY8 epitope but revealed three mutations flanking the VY8 epitope: R⁶⁹K, A⁸¹G, and H⁸⁷R (the VY8 maps to residues 72–79 in this isolate; Fig. 2). At 35 DFOS, 42 clones were sequenced. Fifteen of these contained wild-type flanking regions, whereas 26 possessed the three mutations of interest. In one clone the VY8 epitope was deleted. At 58 DFOS, no wild-type sequence was detectable: all of the sequenced clones ($n = 19$) contained the three substitutions (Fig. 1).

These results indicate a temporal relationship between the loss of specific cytolytic activity and the emergence of proviral sequences encoding mutations flanking the VY8 epitope. This raised the question as to whether these mutations could alter the processing of the epitope and so allow for viral immune escape. We analyzed whether the mutant form of Nef prevents the presentation of the HLA B*35-restricted Nef epitope to VY8-specific CTL. To this end, we constructed rVVs containing full-length wild-type (R⁶⁹, A⁸¹, and H⁸⁷) and mutant (K⁶⁹, G⁸¹, and R⁸⁷) forms of Nef, cloned directly from SC1 (Fig. 2).

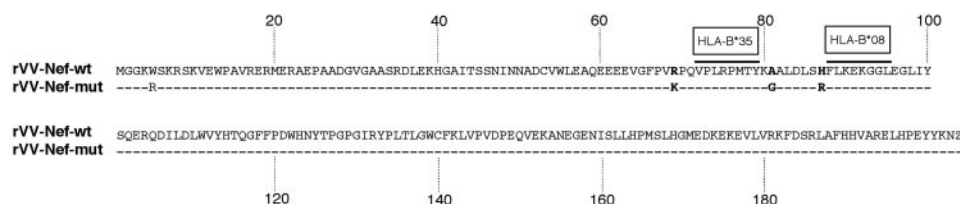


FIGURE 2. Amino acid sequences of the wild-type and mutant Nef protein isolates from SC1 inserted into rVV vectors. The rVV-Nef-wt contains the original Nef isolate (R⁶⁹, A⁸¹, H⁸⁷), and rVV-Nef-mut contains the mutant isolate (K⁶⁹, G⁸¹, R⁸⁷). The three flanking mutations are shown in bold. Epitopes VPLRPMTY (HLA B*35-restricted) and FLKEKGGGL (HLA B*8-restricted) are marked.

The VY8-flanking mutations are associated with poor presentation of the VY8 epitope

To investigate any differences in the presentation of the HLA B*35-restricted Nef VY8 epitope from the wild-type and mutant isolates from SC1, we infected HLA B*35⁺ B cells with the wild-type (rVV-Nef-wt) and mutant (rVV-Nef-mut) Nef constructs, allowed them to express Nef overnight, and used them as targets for specific CTL lines in a 16-h ⁵¹Cr-release assay. The proportion of lysed targets infected with the rVV-Nef-wt construct was ~55% higher than of those expressing the mutant construct (35 vs 19% at E:T ratio of 20:1) (Fig. 3).

A reduction in the abundance of the VY8 epitope derived from the mutant Nef rVV could result from several causes: differential Nef expression levels between rVV-Nef-wt and rVV-Nef-mut, an inherent difference in stability between the two Nef isolates, reduction in cell surface HLA class I expression (and hence Ag presentation) induced by Nef (52–54), or a VY8 epitope processing impairment caused by the VY8-flanking mutations. To rule out the first possibility, we analyzed the expression levels of the two rVV Nef constructs by Western immunoblotting and found no difference in the expression levels (Fig. 4A, inset). The stabilities of the wild-type and mutant Nef isolates were tested using the pulse-chase method and revealed no significant difference between their half-lives (Fig. 4A). The levels of HLA class I expression on the B cell surface following infection with the two Nef rVVs were identical, as measured by FACS and a FITC-conjugated anti-human HLA class I pan-specific Ab W6/32 (Serotec) (Fig. 4B). Furthermore, addition of exogenous peptide to HLA B*35⁺ BCLs, with or without rVV infection, led to similar levels of maximal lysis (Fig. 3), which is also consistent with comparable degrees of class I surface expression.

To confirm that the difference in peptide abundance as processed from the rVV-Nef-wt and rVV-Nef-mut was characteristic of the VY8 epitope alone, we used a neighboring HLA B*08-restricted Nef FLKEKGGL (FL8) epitope as a control. The FL8 epitope maps eight residues downstream of VY8 (Fig. 2). EBV-transformed B cells from a HLA B*08⁺/B*35⁺ individual were infected with either rVV-Nef-wt or rVV-Nef-mut, incubated overnight for Nef expression, and used as targets for VY8-specific and

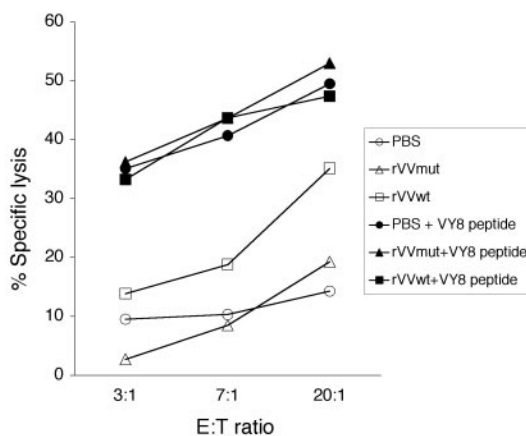


FIGURE 3. Presentation of the HLA B*35-restricted Nef VY8 epitope from wild-type and mutant vaccinia. Autologous BCL were infected with either wild-type (rVV-Nef-wt) or mutant (rVV-Nef-mut) vaccinia, allowed to express overnight, and used as targets for a Nef VY8-specific heterologous CTL clone in a 16-h ⁵¹Cr-release assay. Maximal lysis was obtained with the addition of exogenous peptide (positive control).

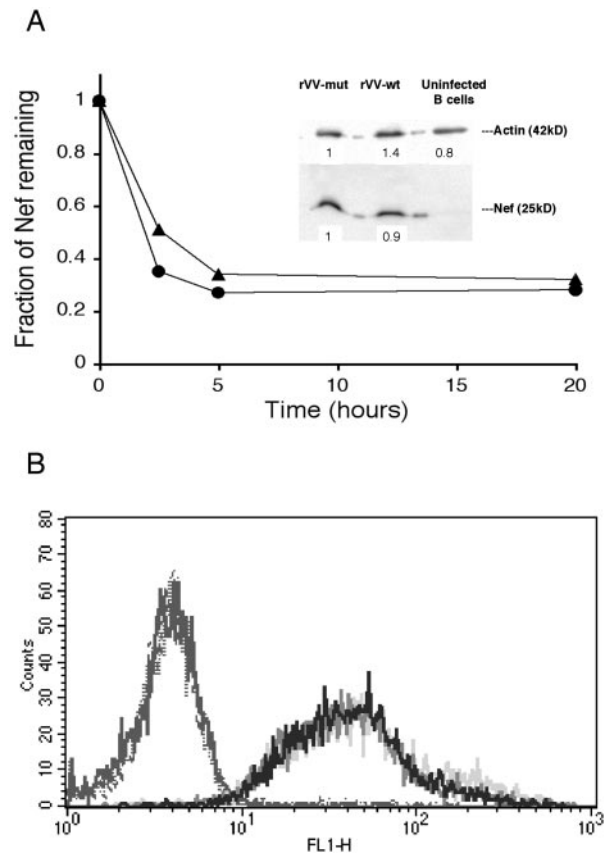


FIGURE 4. A, Expression levels and stability of the mutant and wild-type Nef isolates from SC1. Pulse-chase analysis measuring the intracellular rate of degradation of Nef expressed from the wild-type and mutant rVV constructs (▲, rVV-wt; ●, rVV-mut). Approximately 30–40% of the Nef protein from both constructs was stable for over 20 h. Inset, Western immunoblot of the lysate of B cells infected with mutant or wild-type rVV-Nef and probed with sheep anti-Nef polyclonal Ab ARP444. Blots were also probed with anti-actin Ab as a protein loading control. Numbers denote the relative amounts of protein in each band. B, HLA class I expression levels on B cells following rVV-Nef-wt and rVV-Nef-mut infection. Autologous B cells were infected with rVV-Nef-wt and rVV-Nef-mut, allowed to express for 16 h, stained with FITC-conjugated anti-HLA class I Ab W6/32 and analyzed by FACS. Black trace shows rVV-Nef-mut and gray trace rVV-Nef-wt. The FITC-negative peak is the isotype control staining.

FL8-specific CTL. There was ~60% decrease in the number of VY8-specific CTL producing IFN- γ in response to the rVV-Nef-mut ($p < 0.013$; Student's t test). There was no difference in the recognition of the FL8 epitope (Fig. 5). These findings indicate that the R⁶⁹K, A⁸¹G, and H⁸⁷R mutations interfere specifically with the processing and presentation of the VY8 epitope; the FL8 epitope also provides an internal control for Nef expression and processing from the two constructs.

*The HLA B*35-restricted Nef VY8 epitope is generated by the proteasome*

A previous study has indicated that the HLA B*35 Nef VY8 epitope is processed by the proteasome (55). We confirmed this finding by using a rVV encoding a ubiquitinated form of Nef, rVV-UbRNef (56), which is rapidly degraded by the 26S proteasome. In a 16-h ⁵¹Cr release CTL assay, following an overnight

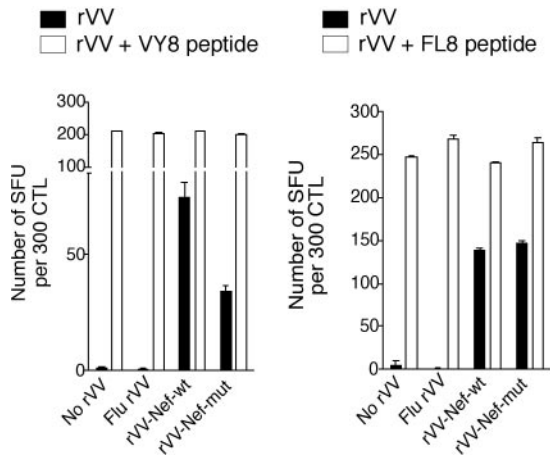


FIGURE 5. Comparison between Nef VY8 and Nef FL8 epitope presentation from rVV-Nef-wt and rVV-Nef-mut. HLA B*35⁺/HLA B*08⁺ heterologous BCL were infected with rVV-Nef-wt or rVV-Nef-mut and used as targets in a 4-h ELISPOT assay with either VY8-specific (HLA B*35-restricted) or FL8-specific (HLA B*08-restricted) CTL. The FL8 epitope served as an internal control for variables unrelated to the processing of the Nef VY8 epitope. Each assay was conducted in quadruplicate with 300 CTL/well. An irrelevant recombinant vaccinia containing the *matrix* gene from the influenza virus (rVV-flu) was used as a negative control.

rVV-UbRNeF expression, at 20:1 E:T ratio, lactacystin-treated infected cells were recognized nearly 65% less efficiently than untreated infected cells (Fig. 6).

To examine further the processing of VY8 epitope and the potential involvement of other Ag-processing mechanisms, we tested the intracellular effect of several protease and proteasome inhibitors on the presentation of VY8 from rVV-Nef-wt. In an overnight rVV expression assay, followed by ELISPOT (as described above), the addition of 10 μ M Epoxomicin, a highly specific proteasome inhibitor, almost completely abolished the presentation of the VY8 epitope (Fig. 7A). A similar effect was observed with 5–10 μ M MG-132, an inhibitor of the proteasome and cysteine proteases (Fig. 7B). To test whether the effect of MG-132 on VY8 presentation indeed occurs through proteasome inhibition, we used an inhibitor of cysteine proteases, EST (E64d). Even at the highest concentration recommended by the manufacturer (100 μ M), EST did not interfere substantially with VY8 presentation, indicating a lack of involvement of cysteine proteases (Fig. 7C).

An important proteasome-independent mechanism of Ag processing has recently been assigned to the TPP II (51, 57). We used a TPP II inhibitor AAF-CMK to investigate the involvement of TPP II in the processing and presentation of VY8. No significant effect was observed at concentrations up to 50 μ M AAF-CMK (Fig. 7C). Altogether, these data strongly suggest that the proteasome is the principal processing apparatus involved in the presentation of the Nef VY8 epitope.

R⁶⁹K, A⁸¹G, and H⁸⁷R flanking mutations interfere with the proteasomal processing of the VY8 epitope in vitro

To investigate whether the R⁶⁹K, A⁸¹G, and H⁸⁷R flanking mutations influence the efficiency of the proteasome in processing the VY8 epitope, we conducted in vitro proteasomal cleavage of two 25-mer oligopeptides, one corresponding to the wild-type SC1 Nef 66–90 (SC1-Nef-wt^{66–90}) and the other to the triple-mutant (SC1-Nef-mut^{66–90}) sequence from patient SC1. Digests were conducted

with both the immuno-20S and constitutive-20S proteasome. Aliquots were collected at 0, 2, 6, 12, 18, and 24 h of digestion. Most of the digestion fragments were already present after 2 h. After 6 h of proteasomal digestion, the original 25-mer oligopeptide sequence was no longer detectable. After 24 h, some of the earlier digestion products had been degraded further, possibly due to the re-entry of the primary digestion products into the proteasome (21). The in vitro cleavage footprints of the c20S and i20S proteasomes detected after 6 h of proteolysis of the wild-type and mutant Nef oligopeptides are illustrated in Fig. 8.

In vitro digestion of the wild-type oligopeptide demonstrated no difference in the digestion pattern between c20S and i20S proteasomes. Because mass spectrometry is only semiquantitative, it is difficult to ascertain the relative ratios of the excised fragments. Nevertheless, we noted that the dominant cleavage product (i.e., the highest peak at the majority of time points) of the SC1-Nef-wt^{66–90} 25-mer produced by both proteasomes was the F⁶⁶-Y⁷⁹ fragment, which is the correct C-terminal for the VPLRPMTY epitope. The remaining fragment of the original 25-mer (K⁸⁰-K⁹⁰) was also readily detectable (data not shown).

In the SC1-Nef-mut^{66–90} oligopeptide, however, the i20S proteasome produced a novel cleavage point between M⁷⁷ and T⁷⁸, whereas the c20S proteasome cleaved the epitope at two other sites (R⁷⁵/P⁷⁶ and P⁷⁶/M⁷⁷; Fig. 8). Thus, both forms of the 20S proteasome disrupt the VY8 epitope when flanked by the ⁶⁹K, ⁸¹G, and ⁸⁷R variant residues. It is highly likely that the reduced recognition of the VY8 epitope by VY8-specific CTL in the in vivo rVV-Nef-mut processing assays (Figs. 3 and 5) can be accounted for by this observation. However, the correctly excised F⁶⁶-Y⁷⁹ fragment was also detected following the digestion of SC1-Nef-mut^{66–90} by both i20S and c20S, which could explain the reduced, but nevertheless detectable, CTL recognition of VY8 (Fig. 5). In

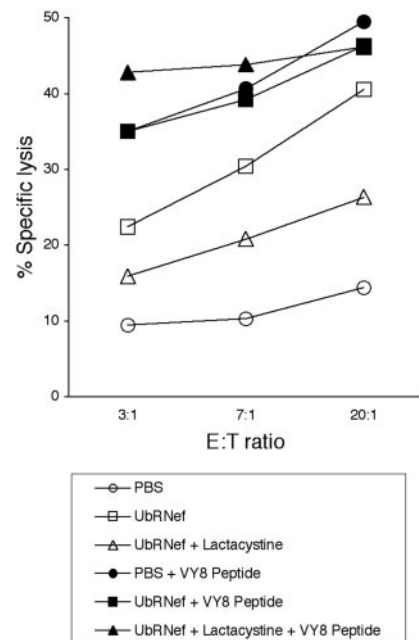


FIGURE 6. Inhibition of lysis of rVV-UbR-Nef-infected targets following lactacystin treatment. EBV-transformed autologous B cells were infected with rVV-UbRNeF (which encodes a ubiquitinated, proteasome-sensitive form of Nef) in the presence or absence of 100 μ M lactacystin, incubated overnight (without lactacystin), and used as targets for a heterologous Nef VY8-specific CTL clone in a 16-h ⁵¹Cr-release assay. VY8 peptide was added at 1 μ M final concentration to parallel control assays.

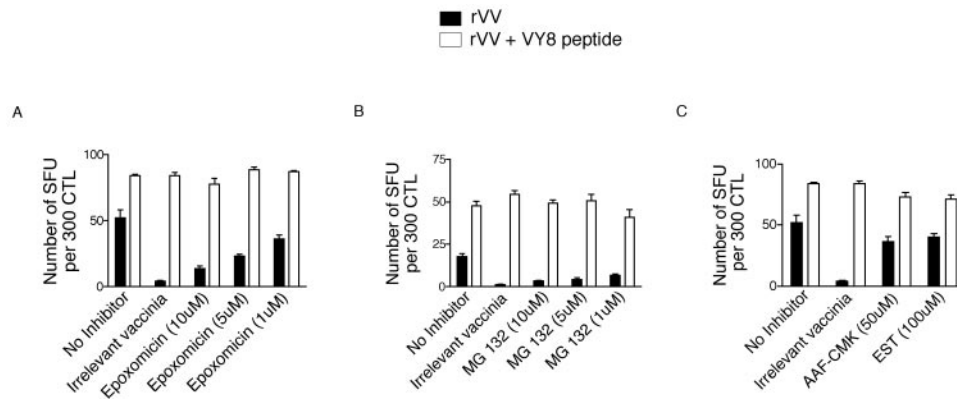


FIGURE 7. Intracellular inhibition of Nef VY8 epitope presentation. Proteasome and protease inhibitors were added to heterologous HLA B*35⁺ BCL at different final concentrations (as indicated), before infection with rVV-Nef-wt. The cells were incubated in 20-fold dilution R-10 medium overnight before being used as targets for heterologous Nef VY8-specific CTL in a 4-h ELISPOT assay. All used inhibitors are irreversible apart from MG-132, which was hence also added to the cells overnight at the corresponding final concentration. To insure equal experimental conditions for all assays, we performed cell count with trypan blue exclusion and added the same number of live target cells (35,000) in each well. □, Represent the same infected samples with exogenously added VY8 peptide, and serve as a control for the number of live target cells in each well. *A*, Epoxomicin, proteasome-specific inhibitor. *B*, MG-132, proteasome and cysteine proteases inhibitor. *C*, AAF-CMK, inhibitor of TPP II protease; EST, cysteine proteases inhibitor (maximal tested concentrations).

short, our *in vitro* processing data strongly suggest that the presence of R⁶⁹K, A⁸¹G, and H⁸⁷R mutations leads to the altered cleavage of Nef VY8 by both the constitutive and the immunoproteasome, resulting in the partial destruction of this epitope.

Discussion

We detected a HIV Nef variant that rose to fixation in the face of a CTL response to a HLA B*35-restricted epitope in a patient with primary HIV-1 infection. The variant form of Nef contained R⁶⁹K, A⁸¹G, and H⁸⁷R mutations, which flank the HLA B*35-restricted VY8 epitope. This protein had a reduced capacity to activate CTL specific for the VY8 epitope, but not CTL that recognized a distinct epitope restricted by HLA B*08. Intra-epitope proteasomal cleavage of VY8 dictated by the mutated flanking residues was shown to be the likely cause for the reduced epitope presentation. This finding substantiates the concept of HIV immune evasion due to genetic variation that alters the processing of a CTL epitope.

The origin of the HIV Nef variant in patient SC1 is unclear. The first sampling time point was several weeks into the infection; studies of SIV in rhesus macaques have shown escape mutations as early as 4 wk postinfection (7), so the mutations may have arisen *de novo* in this patient, either at random or possibly to compensate for fitness impairing mutation(s) elsewhere in the protein (14, 58). It is also possible that some of the three mutations arose due to immune pressure from other HLA class I alleles (34), because CTL epitopes cluster densely in this region of Nef (www.hiv.lanl.gov). Alternatively, the mutant form of Nef may have been transmitted to SC1 and subsequently fixed under the selection pressure exerted by patient's CTL, because the mutations enabled immune escape (59). The parallel emergence of three clustered variant residues in this patient strongly supports the last possibility. In addition, review of the literature and the HIV sequence database (www.hiv.lanl.gov) reveals that ⁸⁷R is rare, whereas ⁶⁹K and ⁸¹G commonly appear in Nef sequences from HIV patients, although not together (60, 61). It is feasible that recombinant events and/or mutations arising on the HIV backbone with one or two of these variants have resulted in the cumulative triple mutant virion that infected SC1. The func-

tional significance of these variant residues in HIV pathogenesis has not been previously investigated.

This study has demonstrated that the R⁶⁹K, A⁸¹G, and H⁸⁷R variant created a novel intraepitopic cleavage site in Nef and interfered with presentation of a HLA class I-restricted epitope without compromising the expression and stability of the Nef protein or affecting cell surface HLA class I expression in infected cells. Our findings are in accord with an earlier study on HIV-1 Nef; epitope QVPLRPMTYK (QK10), corresponding to residues 71–80 of SC1 Nef, is HLA A*11-restricted (40) and overlaps with the HLA B*35-restricted VY8 epitope studied here. In a study by Couillin et al. (62), a HLA A*11⁺ HIV-infected patient with Nef sequences bearing ⁶⁹R or ⁶⁹T and ⁸¹A had an efficient response to the QK10 epitope, while another HLA A*11⁺ patient, where these residues had mutated into ⁶⁹K and ⁸¹G (as in SC1), lacked a QK10-specific CTL response. The mechanism for the observed immune escape in the second patient was not investigated.

The N-terminal region of the VY8 epitope studied here contains a well-described Nef PxxP motif, a binding domain for serine/threonine (63), tyrosine (42), and Src kinases (64). This motif is highly conserved and has previously been reported as crucial in controlling viral entry, replication and progression to AIDS or simian immunodeficiency syndrome (65–67). Consequently, this region of Nef is under biological constraints that limit tolerance of mutations within the epitope itself. In such a situation, mutations outside the epitope that interfere with its processing could provide an alternative form of immune escape, as we have demonstrated.

The presence of R⁶⁹K, A⁸¹G, and H⁸⁷R flanking mutations led to the loss of the HLA B*35-restricted Nef VY8 epitope CTL response in patient SC1 as a result of impaired Ag processing from the variant Nef. A previous study has shown that the proteasomal cleavage footprint of HIV Nef corresponds to the carboxyl end of the VY8 epitope eluted from the cell surface, indicating that this epitope is processed by the proteasome (55). Our data confirms this finding and further shows a lack of involvement of other proteolytic mechanisms (cysteine proteases, TPP II) in the processing and presentation of VY8. In the presence of R⁶⁹K, A⁸¹G, and

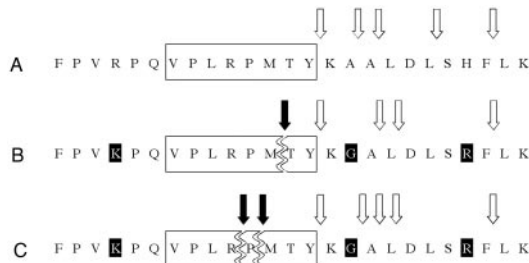


FIGURE 8. In vitro cleavage patterns of mutant and wild-type Nef sequences from patient SC1 obtained using the constitutive and the immuno-20S proteasome. The 25-mer oligopeptides corresponding to the wild-type and mutant SC1 Nef 66–90 were digested in vitro using the constitutive and immuno-20S proteasomes. Arrows indicate cleavage sites for wild-type 25-mer with c20S or i20S (A), mutant 25-mer with i20S (B), and mutant 25-mer with c20S proteasome (C). Black arrows show cleavage points within the VY8 epitope introduced by the presence of the flanking mutations.

H⁸⁷R, the constitutive and immunoproteasome demonstrated altered cleavage patterns within the epitope, each resulting in the disruption of the epitope.

Differences in the cleavage motifs of the c20S and i20S have been previously documented in other systems (68, 69). Apart from the cleavage through the epitope, both forms of the proteasome also produced a correctly excised C terminus of the VY8 epitope. These data correlate well with the reduction in the release of the VY8 peptide from the mutant form of Nef. However, because it is not possible to draw parallels between in vitro proteasomal digestion and in vivo effects of differential cleavage on viral presentation, the observed partial epitope destruction and presentation may have a significantly higher impact on viral immune evasion in vivo.

Several studies have shown a correlation between the HLA B*35 class I allele and rapid progression to AIDS (70–72), which may be attributable to a low efficiency in presenting epitopes. Viral escape has also been linked with progression to AIDS (73, 74). Our study is consistent with both, because it demonstrates HIV escape through an epitope-processing mutation within a conserved genomic region as well as reduced wild-type VY8 epitope presentation by the HLA B*35 allele, at least when compared with the HLA B*08-restricted FL8 epitope (Fig. 5).

Compared with the large number of studies describing viral escape by mutation of anchor residues for MHC binding or disruption of T cell recognition, the reports of immune escape through epitope-flanking mutations have been few and recent (32–34). One possible explanation for this paucity is that a single polymorphism can completely abolish MHC binding of the epitope or abort TCR recognition (13, 75). In contrast, unless a mutation fully blocks processing and presentation of the epitope, a flanking variant may cause an intermediate phenotype. If some residual peptide is liberated by the novel processing, viral recognition by high-avidity CTL may still occur. Nevertheless, single amino acid variants resulting in reduced surface presentation of the epitope could be very important in shaping the adaptive intrahost evolution of the virus.

In conclusion, the emergence of Nef VY8 epitope-flanking mutations described in this study led to the waning of a strong CTL response to VY8 in the patient, as a consequence of viral immune escape through proteasomal epitope disruption, while preserving Nef expression and stability. These results substantiate the importance of epitope-flanking mutations in viral pathogenicity (33, 34). The relative importance of this mode of immune escape in HIV

infection remains uncertain. However, the extent of viral escape may be considerably underestimated if only within-epitope variations are investigated when documenting viral escape from immune surveillance (7).

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Disclosures

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